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HAZARD EVALUATION DIVISION

STANDARD EVALUATION PROCEDURE

AVIAN DIETARY LC_{50} TEST

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16. Abstract (Limit: 200 words)

The Standard Evaluation Procedure (SEP) for the Avian Dietary LC50 Test is a guidance document primarily intended for Agency reviewers and the regulated industry who evaluate ecological effects data specified in 40 CFR Part 158.145. The SEP is also intended to provide information to the general public indicating how the Agency evaluates these types of studies. As such, it is designed to supplement Subdivision E of the Pesticide Assessment Guidelines: Hazard Evaluation - Wildlife and Aquatic Organisms. This SEP provides an Introduction, Materials and Methods, Reporting Requirements, Reviewer Evaluation, and Appendix of appropriate methods to guide the review and scientific evaluation of pesticide effects on birds.

The SEP for Avian Dietary LC50 Test is only one of a number of SEP's published by the National Technical Information Service as a supplement to Subdivision E of the Pesticide Assessment Guidelines.

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STANDARD EVALUATION PROCEDURE

PREAMBLE

This Standard Evaluation Procedure (SEP) is one of a set of guidance documents which explain the procedures used to evaluate environmental and human health effects data submitted to the Office of Pesticide Programs. The SEPs are designed to ensure comprehensive and consistent treatment of major scientific topics in these reviews and to provide interpretive policy guidance where appropriate. The Standard Evaluation Procedures will be used in conjunction with the appropriate Pesticide Assessment Guidelines and other Agency Guidelines. While the documents were developed to explain specifically the principles of scientific evaluation within the Office of Pesticide Programs, they may also be used by other offices in the Agency in the evaluation of studies and scientific data. The Standard Evaluation Procedures will also serve as valuable internal reference documents and will inform the public and regulated community of important considerations in the evaluation of test data for determining chemical hazards. I believe the SEPs will improve both the quality of science within EPA and, in conjunction with the Pesticide Assessment Guidelines, will lead to more effective use of both public and private resources.

John W. Melone, Director
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AVIAN DIETARY LC50 TEST

I. INTRODUCTION

A. When Required

Avian dietary toxicity tests are required to support registration of an end-use product intended for outdoor application, and to support registration of a manufacturing-use product which may be used to make such an end-use product. These studies are also required, on a case-by-case basis, to support the registration of an end-use product intended solely for indoor application, and to support registrations of manufacturing-use products which may be used to make such an end-use product.

B. Purpose

Avian dietary toxicity tests determine the median lethal concentration (LC_{50}) of a chemical. This is defined as the quantity of toxicant in the diet calculated to kill fifty percent of the test population. These tests have attained broad acceptance among environmental toxicologists as relatively rapid, uncomplicated, inexpensive, and statistically reliable methods for assessing short-term, adverse effects of chemicals on avian wildlife.

The Ecological Effects Branch regularly requires that results of two avian dietary tests (one upland game bird and one waterfowl) be submitted to support the registration of a pesticide. The data from this test are used to:

- Establish acute toxicity levels of the active ingredient to nontarget avian wildlife;
- Compare toxicity information with measured or estimated pesticide residues in the terrestrial environment in order to assess potential impact on avian wildlife;
- Provide support for precautionary label statements to minimize adverse effects to avian wildife; and
- Indicate the need for further testing and/or field studies.

C. Test Substance

1. Technical Grade

Routine tests must be conducted with the technical grade of the active ingredient (a.i.). If more than one a.i. constitutes a technical product then the technical grade of each a.i. must be tested separately.

2. Formulated Product

The Pesticide Assessment Guidelines, Subdivision E, indicate when formulated products should be used in this test. Normally there will be a specific Agency requirement when such testing is called for.

II. MATERIALS AND METHODS: TESTING STANDARDS/RECOMMENDATIONS

A. Acceptable Protocols

Because the dietary test is an established technique for assessing toxicity of a chemical to avian wildlife, much of the methodology for performing these studies, as well as the procedures for statistical analysis of results, have been carefully outlined and documented in the published literature. Notably, the information to be discussed in this Standard Evaluation Procedure (SEP) is presented in greater detail in the following two references:

ASTM. 1980. Standard Practice for Conducting Subacute Dietary Toxicity Tests with Avian Species. ASTM Designation E 857-81.

Ecological Effects Branch. 1982. Pesticide Assessment Guidelines Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, EPA-540/9-82-024. pp. 37-43.

B. Test Organisms

1. Acceptable Species/Age/Physical Condition

Testing must be done on both a wild waterfowl species, preferably mallard duck (Anas platyrhynchos), and an upland game species, preferably bobwhite quail (Colinus virginianus). The age of mallard ducks should be from five to ten days old at the beginning of the test; the 5 day old birds are preferred. Bobwhite quail should be from 10-14 days old at the beginning of the test.

Test birds should be pen-reared and phenotypically indistinguishable from wild birds. It is recommended that the birds to be used should only be from colonies that have had breeding histories maintained for them.

Sickness, injuries, excessive mortality of hatchlings, or any abnormal observations of birds are all signs that the affected lot should not be used for testing.

These species were chosen because of their commercial availability, ecological significance, broad geographical distribution,

commercial and recreational importance, ease of handling in the laboratory, and their past use in toxicity testing and known susceptibility to chemical exposure. Other species may be acceptable on a case-by-case basis; however, this is discouraged, unless specifically required by the Agency. The test species should be verified by its scientific name.

2. Source/Acclimation

Within a given test all organisms must be from the same source (this includes laboratory or commercial stocks) and most preferably from the same hatch. Test birds should be preconditioned to the test facilities and fed untreated test diet for as long as possible prior to the beginning of the test.

C. Test Conditions

1. Number Per Level

The actual dose levels for the "definitive" study should be determined by "range finding" studies. Results from "range finding" studies may not, however, be used to calculate the LC_{50}. Except in cases where the LC_{50} is demonstrated to be greater than 5000 ppm, definitive studies must use a minimum of four treatment levels, but five or six are strongly recommended, plus additional groups for control or vehicle control if a vehicle is used. Ten birds per concentration and ten birds for each control and vehicle control group are strongly recommended. Concurrent control and vehicle control groups are required for each LC_{50} test. In all cases the birds must be randomly assigned to the pens.

Factors between dose levels chosen for the definitive studies should be based on a geometric scale, and using results from range finders, should attempt to produce mortality ranging from 10 to 90 percent. In order to provide statistically reliable results the design of the definitive study should attempt to produce three "partial kills" (i.e., between 0 and 100 percent) surrounding the estimated LC_{50}.

Studies should be designed to establish an actual LC_{50} and 95% c.i.. In lieu of this, a study may demonstrate that the LC_{50} is greater than 5000 ppm. In this case the study should show, on the basis of ten or more birds per dose, that less than one-half of the birds died at 5000 ppm. When any mortality is observed at 5000 ppm, sequentially lower levels must be tested (with ten birds per level) in order to get a dose-response series which includes at least one "no-effect" (no mortality) level.

2. Pen Facilities

Pen conditions should conform to good husbandry practices. For example, bobwhite quail (about ten birds per pen) can be tested in commercial brooder units with individual pens about 35 x 100 x 24 cm high; mallards should be tested in 70 x 100 x 24 cm high pens. Floors and external walls can be of wire mesh, while ceilings and common walls may be galvanized sheeting. The brooder temperature for both species should be about 35°C. Thermostatic control may be used. Temperatures outside the brooder may range from 22-27°C. The relative humidity should generally be not less than 30 nor more than 80 percent. Adequate ventilation should be maintained.

3. Photoperiod

Lighting may be supplied on a varying regime. A diurnal photoperiod is recommended but 24-hour lighting may also be used (fluorescent or incandescent forms are both acceptable).

4. Body Weights

Individual body weights should be measured at the beginning and the end of the study. Although individual body weight data is preferred, only mean body weights for each test and control group are required to be reported for the initiation and termination of the test.

5. Food Consumption

Food consumption must be recorded at the beginning and end of the pretreatment, treatment, and observation periods. Estimates of average consumption for each pen must be reported. Provisions for minimizing food spillage and preventing air contamination by volatile chemicals should be reported.

6. Dose Preparation

A standard commercial game bird or waterfowl diet (mash) should be used to prepare treated diets. Except for dry material, the test substance should be added to the diet without a vehicle, if possible. If necessary, the preferred vehicle is distilled H_2O. If an evaporative vehicle is used (such as acetone or methylene chloride) in preparing water insoluble material, it should be completely evaporated at room temperature prior to feeding. Acceptable vehicles include corn oil, propylene glycol, 1% carboxymethylcellulose, and gum arabic. Maximum vehicle amount per diet should not exceed 2% of the diet by weight. Control diets must contain the maximum amount of vehicle available to treatment birds. Diets and toxicants should be freshly mixed in small batches and then added to larger quantities of diets in order to

achieve a uniform mix of the toxicant in the final diet. Water should be freely available throughout the test.

7. Diet Admininstration/Test Duration

The test is divided into two phases for a total of eight days. Phase I - five day "treated" diet available to experimental birds ("clean" or untreated diet for control groups); Phase II - three day observation period when "clean" food is available to both experimental and control groups. The observation period may have to be extended beyond three days, e.g., for certain chemicals such as anticoagulants, where delayed mortality may be observed on the last few days of the study. In such cases observation must be continued until there is no mortality for at least seventy-two consecutive hours. Control mortality must not exceed ten percent during the same period.

III. REPORTING REQUIREMENTS

A. Test Material

The purity of the test material must be stated and should generally be "technical" grade unless otherwise required. The percent a.i. must be stated.

B. Observable Effects Criteria

The criteria for determining effects must be defined. The raw data or percentage of deaths/effects at each test level and control as well as the number of birds tested per group must be reported.

C. Calculated LC50

The statistically calculated LC_{50} with 95% confidence interval and the method of calculation must be presented. In lieu of a calculated LC_{50}, the study may show that the LC_{50} is greater than 5000 ppm. The slope of the dose-response line should be calculated and reported. Some reports have a graphic (log probit/dose level) presentation which is helpful.

D. Results of Chemical Analysis

If the concentration of the test material was measured, the results should be reported. It is particularly important that measured concentrations are presented if:

- The test material was a dry powder and no vehicle was used; or
- The test material was volatile.

E. Body Weight and Food Consumption

The individual body weights are preferred but the report need only provide mean body weights and mean food consumption for each test and control group at the beginning and end of each phase of the test.

F. Raw Mortality Data

Raw mortality data or percentage of death/effects must be reported. The data must indicate the numbers of birds dying at each dose or control level. The day of death/effects must be reported; it is preferable to also report the time of death.

G. Gross Necropsy

Gross necropsies are preferred. When performed, all dead birds should be examined as well as a sufficient number of survivors in order to provide a characterization of gross lesions. Inspections of the GI tract, liver, kidneys, heart, and spleen should be made. Also, examinations of subcutaneous fat for muscle deterioation should be made.

H. Other Observations

Signs of intoxication should be described as to what was observed, when, and for how long. Technical terminology used to name signs of intoxication should be adequately defined if these terms are not in general use. The report should indicate whether deaths are associated with specific signs of intoxication, and the time of onset and remission of toxic signs. If affected birds recover, the time interval to recovery should be reported. Marked observations of fecal material and urine should be reported.

I. Statistical Analysis

A statistical analysis of the mortality data is required. The LC_{50}, 95% confidence intervals, and slope of the dose-response line should be calculated using one of the methods referenced in the Pesticide Assessment Guidelines, Subpart E. Other statistically valid methods available such as computer software are acceptable, subject to validation by the Agency. When reporting the LC_{50} and 95% c.i. the slope of the dose-response line should be given (the actual dose-response plot need not be included).

J. Acceptable Protocols

EEB does not endorse any one protocol. It is sometimes necessary and desirable to alter the procedures presented in published protocols to meet the needs of the chemical or test

organisms used. However, EEB does recommend some protocols as guidance for developing avian dietary toxicity tests. These protocols include:

ASTM. 1980. Standard Practice for Conducting Subacute Dietary Toxicity Tests with Avian Species. ASTM Designation E 857-81.

Ecological Effects Branch. 1982. Pesticide Assessment Guidelines Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, EPA-540/9-82-024. pp. 37-43.

These referenced protocols are presented as flexible guidance to help researchers design scientific protocol and to help the reviewer validate studies. It is important to recognize that avian dietary tests are validated based on whether they fulfill guideline requirements and whether they provide scientifically sound information on the dietary toxicity of the test material to avian wildlife that is useful in risk assessments. This is more important than whether they follow a referenced protocol step-by-step.

IV. REVIEWER EVALUATION

The reviewer should identify each aspect of the reported procedure that is inconsistent with recommended protocol. The significance of these deviations must be determined. The number of deviations and their severity will determine the validity of the study and the interpretation of the results.

A. Review of Materials and Methods

1. Test Substance

When the technical grade is greater than 80% a.i., the reviewer may elect not to adjust the definitive LC_{50} value to reflect actual amount of a.i.. Reviewers should check the accuracy of the reported LC_{50} value by recalculating the doses in ppm a.i. in treated diets.

2. Diet

A review of the diet composition should be made specifically to check for unfamilar ingredients or medications. Any such unfamilar ingredient or suspected additive should be verified by contacting the testing laboratory prior to classifying the study.

3. Body Weight and Food Consumption

Measurements of food consumption, although estimated, are important because the study also can serve as a palatability study (this may be useful in designing reproduction studies), and

because this information can be used to estimate the amount of toxicant consumed by the test birds. Body weight and food consumption data can be used to evaluate effects, if any, on feeding and/or weight change.

Reviewers should take particular note of vomiting and reduced food consumption. Vomiting, although normally encounterd in the LD_{50} test, could occur in the LC_{50} test. Suggestions for dealing with this problem are contained in a companion SEP document on the LD_{50} study.

It is extremely important to realize that fifty percent of ten day old mallard ducklings can survive for five days without eating! Therefore a study with groups of only ten animals each dose may provide irregularities in dose-response data. However, this test can serve as useful palatability information when combined with food consumption data. Normally wild birds will be able to avoid unpalatable food; starvation of young birds unable to obtain palatable food is considered an indirect effect.

4. Raw Mortality Data

The raw mortality data or percentage of death/effects must be checked to insure consistency with the written report. In cases where the two do not agree, the discrepencies must be explained in writing prior to classifying the study.

The control mortality data must be checked. Generally, not more than 10% control mortality will be acceptable.

B. Verification of Statistical Analyses

The reviewer should "validate" the statistical analysis of the data. This may be done using EEB's "Toxanal" or "SAS" computer programs which calculate the LC_{50}, 95% c.i., and the slope, when calculable.

The statistics should be verified particularly if the raw data do not show clear linear dose-response or if the reported LC_{50} and confidence interval do not seem to match the raw data. Any deviation should be noted and an explanation required from the registrant.

Adjustments to the LC_{50} may be made at this time to reflect the reviewer's estimate of the LC_{50}. These may be necessary to account for actual amount of a.i. consumed, (e.g., in cases where the test material a.i. content was lower than 80% or insufficient diet was consumed, or to correct errors in the author's calculations).

C. Discussion of Results

1. Mortality and Behavioral Observations

The reviewer should discuss the results in light of the observations of intoxication. Mortality which cannot be fully attributed to the effects of the test material may be better understood if assessed in light of behavioral observations. Also, signs of intoxication could aid in understanding potential sublethal effects which could affect the birds' ability to develop and survive in the wild.

2. Implications of Dose/Mortality Response

The dose/mortality response enables derivation of such useful information as the LC_{50} and 95% confidence interval as well as the observed NOEL. It may also reveal important characteristics about the toxicity of the test material, such as whether the response is gradual over a wide range of test levels or rapid with a narrow range between the observed NOEL and 100% mortality.

Pertinent data on dose/mortality response should be included in data evaluation records.

3. Gross Necropsy

The results of gross necropsies, when performed, may be helpful in evaluating the study. When the same lesions are found in dead and surviving experimental birds, this may be an indication that a longer observation period may be necessary. If lesions are found in reproductive organs, reproduction studies may be indicated. Lesions found in dead controls are an indication that the lot should not have been used.

4. General Comments

Reviewers should comment on the protocol used as well as the methods or other aspects of the study which are irregular, unfamiliar, or unacceptable. Suggestions for improvements to the protocol can be made, as well as giving rationale for rejecting certain methods or aspects of the study. Of particular importance are the reviewer's comments on any aspect of the study, such as condition of the birds, husbandry practices, dose administration or rejection, toxic symptoms, or environmental conditions which could bear on the interpretation of the results.

D. Descriptive Categorization of Results

The reviewer should indicate what the results were and how much information can be drawn from them. At a minimum, an avian dietary

toxicity test will provide an LC_{50} with 95% confidence intervals. This should allow classifying the test material based on the following scheme:

LC_{50} (ppm)	Category Description
< 50	very highly toxic
51 - 500	highly toxic
501 - 1000	moderately toxic
1001 - 5000	slightly toxic
> 5000	practically non-toxic

These descriptive categories are somewhat arbitrary and are for in-house use only; mainly for inter-chemical comparison. These categories do not necessarily reflect actual environmental hazard to avian wildlife, nor is the descriptive verbiage used in determining risk. The latter task ultimately rests on quantitative toxicity and exposure data.

E. Reviewer's Conclusions

1. Category

The reviewer should indicate under which of the three validation categories the study fits:

° Core: All essential information was reported and the study was performed according to recommended protocols. Minor inconsistencies with standard methodologies may be apparent; however, the deviations do not detract from the study's soundness or intent. Studies within this category fulfill the basic requirements of current guidelines and are acceptable for use in a risk assessment.

° Supplemental: Studies in this category are scientifically sound; however, they were performed under conditions that deviated substantially from recommended protocols. Results do not meet guideline requirements; however, the information may be useful in a risk assessment. Some of the conditions that may place a study in a supplemental category include:

- Unacceptable test species;
- Inappropriate test material;
- Dosage levels tested were less than 5000 ppm but not high enough to produce an effect on the organisms or a precise LC_{50}/EC_{50}; or
- Deviations from recommended diet preparation procedures.

- Invalid: These studies provide no useful information. They may not be scientifically sound, or they were performed under conditions that deviated so significantly from the recommended protocols that the results will not be useful in a risk assessment.

  Examples of studies placed in this category include those where there were problems with volatility of the test material or when a dry chemical was mixed without the use of a vehicle. Unless acceptable chemical analyses of actual toxicant concentrations were performed in studies such as these, the reviewer cannot be sure that test organisms were actually exposed to nominally designated concentrations.

  A study where the test material was not properly identified can also be invalidated.

2. Rationale

Identify what makes the study supplemental or invalid. It may also be necessary to justify a higher category in spite of deviations. That is, a study may have been called core or supplemental even though there were substantial deviations from recommended protocol. While all deviations should be noted, it may be that the deviations did not actually alter the response of the test organism to the test material. The reviewer is expected to exercise judgment in this area.

3. Repairability

Indicate whether the study may be upgraded or given a higher validation category if certain conditions are met. Usually this would involve the registrant submitting more data on the study.

F. References

The reviewer should reference any information used in the validation procedure. This should include protocol documents, statistical methods, or information taken from files of other divisions or branches within HED.